Title: **Methods of Improving the Effectiveness of Transgenic Plants**

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METHODS OF IMPROVING THE EFFECTIVENESS OF TRANSGENIC PLANTS

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/211,585, filed on June 15, 2000, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to transgenic plants and methods of improving the effectiveness of transgenic plants either by topical application of a hypersensitive response elicitor to the transgenic plant or by incorporating into the transgenic plant a transgene encoding a hypersensitive response elicitor.

BACKGROUND OF THE INVENTION

Transfer of genes into plants is an approach being used with increasing frequency to provide useful and advantageous characteristics to crop and ornamental plants that would be difficult or impossible by traditional breeding methods. Transgenic traits can provide the capacity to synthesize specific compounds including vaccines, antibodies, pharmaceutical peptides, plastic, or industrial enzymes or provide improved physical characteristics such as modified fruit ripening, altered fiber properties, enhanced nutrient or dietary fiber content, herbicide resistance, floral color, or better flavor. Other introduced traits are intended to overcome or minimize particular agricultural problems, such as environmental stress, or attack by specific pathogens or pests that prevent maximum yields from being obtained. Transgenic traits that have been commercialized to date have had very specific and limited functions. Many other transgenic traits currently being developed for commercialization or being considered for introduction into crops are similarly limited or specific in their function.

Environmental factors are an important constraint on the yields obtained from transgenic as well as non-transgenic crops. Losses in productivity due

to disease and damage caused by pathogens and pests can prevent the full benefit of a transgenic trait from being realized. Since many transgenic traits have no effect on disease or pest resistance, transgenic plants are typically just as susceptible to loss and damage as non-transgenic plants. Transgenic traits designed to confer resistance to pests or disease are, in general, limited in scope — i.e., they are effective only against specific pests or diseases. Such transgenic plants are as vulnerable to non-target pests and diseases as non-transgenic plants. Moreover, the process of introducing a transgenic trait can on occasion result in a crop plant becoming more susceptible to a particular disease. This was observed for some varieties of insect resistant transgenic cotton that lost resistance to a particular fungal pathogen.

Genetically determined inherent growth characteristics of any transgenic plant impose an additional limitation on the potential for benefit to be gained. Transgenic traits being developed for commercialization or that have been commercialized to date do not affect plant growth properties, so efficacy of the traits is restricted by an upper limit on growth even under ideal growing conditions. In some cases it has been observed that the introduction of a transgene conferring a value-added trait can actually cause a reduction in yield. Such a reduction in yield is known as a yield penalty. Yield penalties are tolerated when the value-added trait results in a net economic gain; however, reducing or eliminating the yield penalty would be a clear benefit.

A practical constraint on realizing the maximal benefit from transgenic traits is imposed by the length of time required to develop a transgenic crop to the commercial stage. By the time a transgenic line reaches commercialization, the germplasm used as the starting material may be five or more years old and be at a disadvantage in terms of yield or resistance to specific diseases or pests relative to new germplasms developed in the intervening years. Therefore, it would be desirable to provide an approach that would maximize the benefits of a value-added trait, overcome the yield penalty caused by introduction of a value-added trait, and more rapidly develop a transgenic crop or ornamental lines. To achieve these objectives using existing methods or strategies would be excessively time consuming, technically complex, and without any guarantee of success.

A conventional breeding program is one approach that could be chosen to attempt to obtain a genetic background exhibiting enhanced growth and resistance to diseases and pests into which transgenic traits could be introduced. Unfortunately, achieving even marginal improvements in any one of these characteristics by classical breeding has become increasingly difficult and time consuming as the remaining amount of untapped genetic resources available within a given crop species becomes smaller. There is also no guarantee that this approach is feasible since it is unknown whether achieving useful improvements in all these characteristics simultaneously is possible by conventional breeding.

An alternate approach, at least in principle, would be to introduce into plants, in addition to a gene conferring a desired value-added trait, an array of genes each with a specific resistance or growth enhancement trait to provide an umbrella of resistance and yield improvement effects. A large number of genes have been identified that encode proteins with potential to provide resistance to specific types or classes of pathogens if expressed in transgenic plants. In principle, assembling multiple resistance genes in a transgenic plant could confer resistance to a broad range of pathogens. Such resistance genes, however, would not alter the inherent growth characteristics of the plants. Candidate genes that would serve to enhance overall growth and yield in concert with resistance genes are not obvious. Successfully producing transgenic crops that express arrays of transgenes would be technically complex and require even longer development times than are already needed for generating transgenic plants with a single transgene. Introduction of arrays of transgenes into the same crop plant is an approach yet to be proven in practice.

The use of chemical supplements, including fertilizers and pesticides, to enhance realization of value-added traits is also undesirable due to direct and lingering environmental impact which the chemical supplements can have on water supplies and other organisms in the food chain.

The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

One method of the present invention is carried out by providing a plant or plant seed including a transgene conferring a transgenic trait to the plant or a plant grown from the plant seed, and applying to the plant or plant seed a hypersensitive response elicitor protein or polypeptide. According to one embodiment, the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby maximizing the benefit of the transgenic trait to the plant or the plant grown from the plant seed. According to another embodiment, the transgenic trait is associated with a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance in the transgenic plant and the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby overcoming the deleterious effect.

Another method of the present invention is carried out by providing a plant cell, transforming the plant cell with (i) a first DNA molecule encoding a transcript or a protein or polypeptide which confers a trait to a plant grown from the transformed plant cell and (ii) a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different than the protein or polypeptide encoded by the first DNA molecule, the transforming being carried out under conditions effective to produce a transformed plant cell, and then regenerating a transgenic plant from the transformed plant cell. According to one embodiment, transforming with the second DNA molecule imparts enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant, thereby maximizing benefit to the plant of the trait conferred by transforming with the first DNA molecule. According to another embodiment, transforming with the first DNA molecule is accompanied by a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance and transforming with the second DNA molecule overcomes the deleterious effect.

Another aspect of the present invention relates to a transgenic plant including a first DNA molecule encoding a transcript or a protein or polypeptide that confers a trait and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide different than the protein or polypeptide encoded by the first DNA molecule. Also disclosed is a transgenic plant seed obtained from the transgenic plant of the present invention.

A further aspect of the present invention relates to a system for use in transforming plants with multiple DNA molecules. The system includes a first DNA construct including a first DNA molecule which confers a trait to a host plant and a second DNA construct including a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide. Also disclosed is an expression system including first and second vectors into which are inserted, respectively, the first and second DNA constructs.

A related aspect of the present invention concerns a DNA construct including a first DNA molecule which confers a trait to a host plant and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide. Also disclosed is an expression system including a vector into which is inserted a DNA construct which includes the first and second DNA molecules.

Yet another aspect of the present invention relates to a transgenic host cell including a first DNA molecule encoding a transcript or a protein or polypeptide that confers a trait to a host plant and a second DNA molecule encoding a hypersensitive response elicitor protein or polypeptide which is different than the protein or polypeptide encoded by the first DNA molecule.

The hypersensitive response elicitor, when expressed in or topically applied to transgenic plants, confers a trait of enhanced growth, stress tolerance, broad insect resistance, and broad disease resistance (see WO 96/39802; WO 98/24297; WO 98/32844; and WO 98/37752, which are hereby incorporated by reference in their entirety). By either (i) simultaneously introducing a value-added trait and a trait for hypersensitive response elicitor expression into a plant line or (ii) topically applying a hypersensitive response elicitor to a transgenic plant line expressing a value-added trait, it is possible to obtain a transgenic plant line from which the maximal benefit of the value-added trait can be realized. For example, value-added traits which offer

strong but limited benefits (e.g., resistance to a particular pathogen) can be fully realized either by transforming the plants with a transgene or DNA molecule encoding a hypersensitive response elicitor or applying the hypersensitive response elicitor to the plants, both of which will further enhance the same trait by imparting broad growth enhancement, stress tolerance, disease resistance, and/or insect resistance. Similarly, value-added traits which result in a concomitant yield penalty can be fully realized either by transforming the plants with a transgene or DNA molecule encoding a hypersensitive response elicitor or applying the hypersensitive response elicitor to the plants, both of which will overcome the yield penalty by imparting broad growth enhancement, stress tolerance, disease resistance, and/or insect resistance. When expression is utilized rather than topical application, a transgenic germplasm that expresses a hypersensitive response elicitor (i.e., already has enhanced disease resistance and yield properties beyond what is available from conventional hybrid lines) can be transformed with a transgene conferring a specific value-added trait. The same can be said for subsequent introduction of a transgene coding for hypersensitive response elicitor expression into a transgenic germplasm that already expresses a specific value-added trait. Any of these approaches will likely minimize or eliminate any disadvantages relative to conventional hybrids. Thus, the present invention provides an efficient and simple approach which allows for maximal realization of value-added traits and avoids the short-comings and uncertainties of conventional breeding programs.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention is a method carried out by providing a plant or plant seed including a transgene conferring a transgenic trait to the plant or a plant grown from the plant seed, and then applying to the plant or plant seed a hypersensitive response elicitor protein or polypeptide. By applying the hypersensitive response elicitor to the plant or plant seed, as discussed *infra*, enhanced growth, stress tolerance, disease resistance, or insect resistance can be imparted to transgenic plants.

According to one embodiment, the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby maximizing the benefit of the transgenic trait to the plant or the plant grown from the plant seed. For example, when the particular value-added trait relates to specific but limited growth enhancement, stress tolerance, disease resistance, or insect resistance of a transgenic plant, this embodiment relates to providing broad growth enhancement, stress tolerance, disease resistance, or insect resistance that complements the specific but limited value-added trait.

According to another embodiment, the transgenic trait is associated with a deleterious effect on growth, stress tolerance, disease resistance, or insect resistance in the transgenic plant and the applying of the hypersensitive response elicitor is carried out under conditions effective to impart enhanced growth, stress tolerance, disease resistance, or insect resistance to the plant or the plant grown from the plant seed, thereby overcoming the deleterious effect. Thus, this aspect of the present invention is directed to overcoming a yield penalty resulting from a value-added trait.

According to this aspect of the present invention, the effectiveness of a transgenic plant is improved (i.e., maximum benefit is realized or the yield penalty is overcome) following application of a hypersensitive response elicitor protein or polypeptide to either a transgenic plant or a transgenic plant seed from which a plant is grown. The hypersensitive response elicitor protein or polypeptide can be any hypersensitive response elicitor derived from bacterial or fungal sources, although bacterial sources are preferred.

Exemplary hypersensitive response elicitor proteins and polypeptides from bacterial sources include, without limitation, the hypersensitive response elicitors from *Erwinia* species (e.g., *Erwinia amylovora, Erwinia chrysanthemi, Erwinia stewartii, Erwinia carotovora*, etc.), *Pseudomonas* species (e.g., *Pseudomonas syringae, Pseudomonas solanacearum,* etc.), and *Xanthomonas* species (e.g., *Xanthomonas campestris*). In addition to hypersensitive response elicitors from these Gram-negative bacteria, it is possible to use elicitors from Gram-positive

bacteria. One example is the hypersensitive response elicitor from *Clavibacter michiganensis* subsp. *sepedonicus*.

Exemplary hypersensitive response elicitor proteins or polypeptides from fungal sources include, without limitation, the hypersensitive response elicitors (i.e., elicitins) from various *Phytophthora* species (e.g., *Phytophthora parasitica, Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, *Phytophthora citrophthora*, etc.).

The hypersensitive response elicitor protein or polypeptide is derived, preferably, from *Erwinia chrysanthemi, Erwinia amylovora, Pseudomonas syringae,* or *Pseudomonas solanacearum.*

A hypersensitive response elicitor protein or polypeptide from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

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Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
1               5                   10                  15
Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
            20                  25                  30
Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
        35                  40                  45
Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
    50                  55                  60
Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
65                  70                  75                  80
Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                85                  90                  95
Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
            100                 105                 110
Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125
Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
    130                 135                 140
Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145                 150                 155                 160
Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
                165                 170                 175
Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
            180                 185                 190
Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
        195                 200                 205
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Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
    210                 215                 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225                 230                 235                     240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
                245                 250                 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
            260                 265                 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
        275                 280                 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
    290                 295                 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305                 310                 315                     320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
                325                 330                 335

Asn Ala
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This hypersensitive response elicitor protein or polypeptide has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains substantially no cysteine. This *Erwinia chrysanthemi* hypersensitive response elicitor protein or polypeptide is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

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cgattttacc cgggtgaacg tgctatgacc gacagcatca cggtattcga caccgttacg    60
gcgtttatgg ccgcgatgaa ccggcatcag gcggcgcgct ggtcgccgca atccggcgtc   120
gatctggtat ttcagtttgg ggacaccggg cgtgaactca tgatgcagat tcagccgggg   180
cagcaatatc ccggcatgtt gcgcacgctg ctcgctcgtc gttatcagca ggcggcagag   240
tgcgatggct gccatctgtg cctgaacggc agcgatgtat tgatcctctg gtggccgctg   300
ccgtcggatc ccggcagtta tccgcaggtg atcgaacgtt tgtttgaact ggcgggaatg   360
acgttgccgt cgctatccat agcaccgacg gcgcgtccgc agacagggaa cggacgcgcc   420
cgatcattaa gataaaggcg gcttttttta ttgcaaaacg gtaacggtga ggaaccgttt   480
caccgtcggc gtcactcagt aacaagtatc catcatgatg cctacatcgg gatcggcgtg   540
ggcatccgtt gcagatactt ttgcgaacac ctgacatgaa tgaggaaacg aaattatgca   600
aattacgatc aaagcgcaca tcggcggtga tttgggcgtc tccggtctgg ggctgggtgc   660
tcagggactg aaaggactga attccgcggc ttcatcgctg ggttccagcg tggataaact   720
gagcagcacc atcgataagt tgacctccgc gctgacttcg atgatgtttg gcggcgcgct   780
ggcgcagggg ctgggcgcca gctcgaaggg gctggggatg agcaatcaac tgggccagtc   840
tttcggcaat ggcgcgcagg gtgcgagcaa cctgctatcc gtaccgaaat ccggcggcga   900
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tgcgttgtca aaaatgtttg ataaagcgct ggacgatctg ctgggtcatg acaccgtgac  960
caagctgact aaccagagca accaactggc taattcaatg ctgaacgcca gccagatgac 1020
ccagggtaat atgaatgcgt tcggcagcgg tgtgaacaac gcactgtcgt ccattctcgg 1080
caacggtctc ggccagtcga tgagtggctt ctctcagcct tctctggggg caggcggctt 1140
gcagggcctg agcggcgcgg gtgcattcaa ccagttgggt aatgccatcg gcatgggcgt 1200
ggggcagaat gctgcgctga gtgcgttgag taacgtcagc acccacgtag acggtaacaa 1260
ccgccacttt gtagataaag aagatcgcgg catggcgaaa gagatcggcc agtttatgga 1320
tcagtatccg gaaatattcg gtaaaccgga ataccagaaa gatggctgga gttcgccgaa 1380
gacggacgac aaatcctggg ctaaagcgct gagtaaaccg gatgatgacg gtatgaccgg 1440
cgccagcatg gacaaattcc gtcaggcgat gggtatgatc aaaagcgcgg tggcgggtga 1500
taccggcaat accaacctga acctgcgtgg cgcgggcggt gcatcgctgg gtatcgatgc 1560
ggctgtcgtc ggcgataaaa tagccaacat gtcgctgggt aagctggcca acgcctgata 1620
atctgtgctg gcctgataaa gcggaaacga aaaaagagac ggggaagcct gtctcttttc 1680
ttattatgcg gtttatgcgg ttacctggac cggttaatca tcgtcatcga tctggtacaa 1740
acgcacattt tcccgttcat tcgcgtcgtt acgcgccaca atcgcgatgg catcttcctc 1800
gtcgctcaga ttgcgcggct gatggggaac gccgggtgga atatagagaa actcgccggc 1860
cagatggaga cacgtctgcg ataaatctgt gccgtaacgt gtttctatcc gcccctttag 1920
cagatagatt gcggtttcgt aatcaacatg gtaatgcggt tccgcctgtg cgccggccgg 1980
gatcaccaca atattcatag aaagctgtct tgcacctacc gtatcgcggg agataccgac 2040
aaaatagggc agtttttgcg tggtatccgt ggggtgttcc ggcctgacaa tcttgagttg 2100
gttcgtcatc atctttctcc atctgggcga cctgatcggt t                     2141
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The above nucleotide and amino acid sequences are disclosed and further described in U.S. Patent No. 5,850,015 to Bauer et al. and U.S. Patent No. 5,776,889 to Wei et al., which are hereby incorporated by reference in their entirety.

A hypersensitive response elicitor protein or polypeptide derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

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Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1               5                   10                  15
Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30
Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45
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Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145                 150                 155                 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
                165                 170                 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
            180                 185                 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
        195                 200                 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
    210                 215                 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225                 230                 235                 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
                245                 250                 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
            260                 265                 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
        275                 280                 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
    290                 295                 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305                 310                 315                 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
                325                 330                 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
            340                 345                 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
        355                 360                 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
    370                 375                 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385                 390                 395                 400

Gly Ala Ala
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